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# EFFECTS OF CO<sub>2</sub> DRIVEN OCEAN ACIDIFICATION ON ONTOGENETIC STAGES OF THE CUTLLEFISH *SEPIA OFFICINALIS*

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EMBRYONIC DEVELOPMENT  
PH REGULATION  
HYPERCAPNIA  
INVERTEBRATE  
CEPHALPOD  
CALCIFICATION  
CLIMATE CHANGE

**ABSTRACT.** – Ocean acidification due to anthropogenic emissions of CO<sub>2</sub> is the new kid on the block of climate change research and has received considerable attention as changes in seawater acidity and carbonate chemistry can severely affect marine organisms from the species to the ecosystem level. The degree of sensitivity within a species can vary greatly along ontogeny, often leading to highest sensitivities in early life stages. Recent studies using the cuttlefish *Sepia officinalis*, demonstrated that it's oviparous developmental mode can constitute an additional challenge for early life stages as increases in environmental pCO<sub>2</sub> add on top to the already high CO<sub>2</sub> concentration inside the egg. The micro-environment inside the egg is characterized by low pH, hypoxia, hypercapnia and high ammonia concentrations as a result of the animal's metabolism and the limited diffusion permeability of the egg capsule. This oviparous developmental mode in combination with lower pH regulatory capacities are probably the major reasons why *S. officinalis* embryos respond more sensitively towards seawater acidification compared to adults. Although stronger hypercapnia levels (> 0.3 kPa; < pH 7.5) could demonstrate potentially negative effects on the development, metabolism and calcification, acidification levels as predicted for the coming century will probably not severely affect *S. officinalis*. This relative tolerance may be a consequence of a lifestyle in benthic coastal habitats, pre-adapting *S. officinalis* to natural fluctuations in environmental pCO<sub>2</sub>.

## INTRODUCTION

### *Ocean acidification effects on marine organisms*

Elevated environmental CO<sub>2</sub> concentrations (hypercapnia) are a stressor that has lately received considerable attention in the context of climate change. Anthropogenic CO<sub>2</sub> emissions are predicted to lead to a rise in surface ocean pCO<sub>2</sub> from 0.04 kPa up to 0.08-0.14 kPa within this century (Orr *et al.* 2005, Cao & Caldeira 2008). The increased hydration of CO<sub>2</sub> changes seawater chemistry, causing a drop in ocean pH, a phenomenon termed “ocean acidification”. Elevations in aquatic pCO<sub>2</sub> and the associated increase in seawater H<sup>+</sup> concentration affect the physiology of water breathing animals as the pCO<sub>2</sub> in body fluids must increase as well in order to maintain a sufficient diffusion gradient for CO<sub>2</sub> from the animal to the seawater (Melzner *et al.* 2009b). However, an increased hydration of CO<sub>2</sub> in body fluids generates protons which can cause an acidosis if not actively compensated (Heisler 1986). Among the aquatic taxa some have been identified as more sensitive (e.g. less active calcifying species such as corals or echinoderms) whereas others (many active species such as adult fish, crustaceans and cephalopods) can seemingly tolerate high CO<sub>2</sub> concentrations over long exposure times (e.g. Spicer *et al.* 2007, Gutowska *et al.* 2008, Melzner *et al.* 2009a, Ries *et al.* 2009, Dupont *et al.* 2010). Sensitivity of marine organisms towards environmental hypercapnia was mainly reflected in reduced

growth and development as well as altered calcification rates and energy re-allocations (Fabry *et al.* 2008, Stump *et al.* 2012). It has been suggested that the degree of tolerance correlates with the ability to compensate for a hypercapnia-induced acidosis by actively accumulating bicarbonate and eliminating protons from body fluids (Melzner *et al.* 2009b). Such regulatory abilities require the presence of an acid-base regulating machinery consisting of ion transporters and channels located in cells of specialized epithelia. However, regulatory capacities and the structure of regulatory epithelia may change along ontogeny leading to a broad range of sensitivities in response to seawater hypercapnia even within one species (Dupont *et al.* 2008, Walther *et al.* 2010, Hu *et al.* 2011b, Stump *et al.* 2011, 2012, Tseng *et al.* 2013). In these studies, larval stages were identified as the weakest ontogenetic link in many species including fish, crustaceans, cephalopods and echinoderms. Even if adults can tolerate elevated water pCO<sub>2</sub>, ecological fitness of larval stages may be compromised, and thus can negatively affect the survival of the species.

### *Sepia officinalis in a changing environment*

Cephalopods have occupied ecological niches in many marine habitats ranging from tropical to polar regions and have a vertical distribution range from surface waters to the deep sea (Nesis 2003). Very different lifestyles have evolved in this animal class with highly active pelagic

forms to sluggish slow moving benthic species. The cuttlefish *Sepia officinalis* has a demersal lifestyle and has evolved a decoupled swimming mode by either using undulatory movements of the lateral fins running along the whole length of the mantle or energetically more expensive jet propulsion (O'Dor 2002). Moreover, in contrast to squids which are negatively buoyant, *Sepia* spp. have a gas filled cuttlebone enabling these animals to control their buoyancy. These features enable *S. officinalis* to have an energetically favorable lifestyle, compared to most pelagic squids, but restricts their life to benthic, coastal habitats (O'Dor 2002).

Already today, many benthic habitats in coastal areas are characterized by strong fluctuations in temperature, as well as  $pO_2$  and  $pCO_2$  due to the microbial degradation of organic matter, leading to naturally acidified and hypercapnic conditions (Feely *et al.* 2008, Thomsen *et al.* 2010, Melzner *et al.* 2013). Accordingly, due to its benthic lifestyle in coastal habitats, it can be hypothesized that *S. officinalis* is probably pre-adapted to environmental fluctuations in  $pO_2$  and  $pCO_2$  to a certain degree compared to most pelagic cephalopods. Particularly during early development, the embryo may experience strong variations in environmental  $pO_2$ ,  $pCO_2$  as the eggs of *S. officinalis* are spawned in shallow coastal areas attached to hard substrate. Similar to many other molluscs, the eggs of *S. officinalis* are laid in dense clusters with up to several hundred eggs generating steep oxygen gradients into egg masses (Cohen & Strathmann 1996). Furthermore hypercapnic and hypoxic conditions naturally occur in eggs of cephalopods and have led to physiological adaptation in early life stages (Gutowska & Melzner 2009, Hu *et al.* 2013, Thonig *et al.* 2014). Despite a potential pre-adaptation of *S. officinalis* to fluctuations in  $pCO_2$ , ocean acidification has been proposed to constitute a significant stressor in benthic systems due to an amplification of hypercapnia by ocean acidification in naturally acidified and hypoxic habitats (Melzner *et al.* 2013). However, the relatively short lifecycle of *Sepia officinalis* (i.e. 1-2 years) offers this species a substantial chance for evolutionary adaptation in times of rapid environmental change. The present review aims at summarizing our present knowledge regarding the effects of climate change relevant hypercapnia levels on different ontogenetic stages and at different levels of physiological organization in the cuttlefish *S. officinalis*.

### Early life stages

#### Effects of sea water acidification on the embryonic micro-environment

The early development of the cuttlefish embryo takes place inside an egg capsule that protects from biotic and abiotic environmental stressors. Egg capsules from marine species typically have diffusion coefficients of only

10-20 % compared to that of pure seawater (Wickett 1975, Cronin & Seymour 2000, Brante 2006). Due to this limited diffusion permeability for gases, the egg capsule of cephalopods, including that of *S. officinalis*, constitutes a diffusion barrier, leading to hypoxic (low  $pO_2$ ) and hypercapnic (high  $pCO_2$ ) conditions inside the egg. The degree of hypoxia and hypercapnia correlates with the aerobic metabolism of the embryo, and increases during development until hatch. For example, studies using *Sepia apama* could demonstrate that towards hatch,  $pO_2$  decreased to 5-6 kPa despite a thinning of the egg capsule to improve gas permeability (Cronin & Seymour 2000). Furthermore,  $pCO_2$  values increase up to 0.3-0.4 kPa in late stage *S. officinalis* embryos due to an enhanced release of metabolic  $CO_2$  (Gutowska & Melzner 2009).  $CO_2$  perturbation experiments demonstrated that environmental hypercapnia is additive to the already high  $pCO_2$  inside the egg capsule of cuttlefish and squid embryos to maintain a sufficient diffusion gradient of  $CO_2$  (Hu *et al.* 2011b, 2013, Dorey *et al.* 2013, Rosa *et al.* 2013). The maintenance of this diffusion gradient of approximately 0.2-0.3 kPa is essential in order to excrete  $CO_2$  from the perivitelline fluid (PVF). For example, the PVF  $pCO_2$  under control conditions (0.04 kPa  $pCO_2$ ) is about 0.2-0.3 kPa (pH 7.4) in late stage embryos and increases to 0.4-0.5 kPa (pH 7.1) when exposed to environmental hypercapnia (0.14 kPa) (Hu *et al.* 2011b, Dorey *et al.* 2013). The cuttlefish embryo seems to be capable of actively controlling the swelling process of the egg enabling a tuning of the egg capsule diffusion permeability. In this respect, two studies (Lacoue-Labarthe *et al.* 2009, Dorey *et al.* 2013) have demonstrated an increased swelling of *S. officinalis* eggs exposed to hypercapnic conditions of 0.14 kPa; a process that could improve  $CO_2$  permeability and reduces partial pressure gradients during environmental hypercapnia. The effects of increased seawater  $pCO_2$  on important abiotic variables in the seawater, inside the perivitelline fluid (PVF) and within the embryo, are summarized in Fig. 1.

#### Growth and development

Particularly in early life stages of marine vertebrates and invertebrates,  $CO_2$  induced seawater acidification has been demonstrated to elicit reductions in developmental rates (Dupont *et al.* 2010, Walther *et al.* 2010, Hu *et al.* 2011b, Stumpp *et al.* 2011, Tseng *et al.* 2013). This phenomenon was also observed in squid and cuttlefish embryonic stages with reductions in developmental rate (indicated by the embryo wet mass) by 16 % (*S. officinalis*) and 28 % (*Sepioteuthis lessoniana*) at an ambient  $pCO_2$  of 0.4 kPa (pH 7.3 to 7.4) compared to animals kept under control conditions at 0.04 kPa (pH 8.1) (Hu *et al.* 2011b, 2013). In *S. officinalis*, no effects on growth and development were observed for moderate hypercapnia levels below 0.14 kPa  $pCO_2$  (Hu *et al.* 2011b, Dorey *et al.* 2013). Interestingly, reductions in growth and devel-

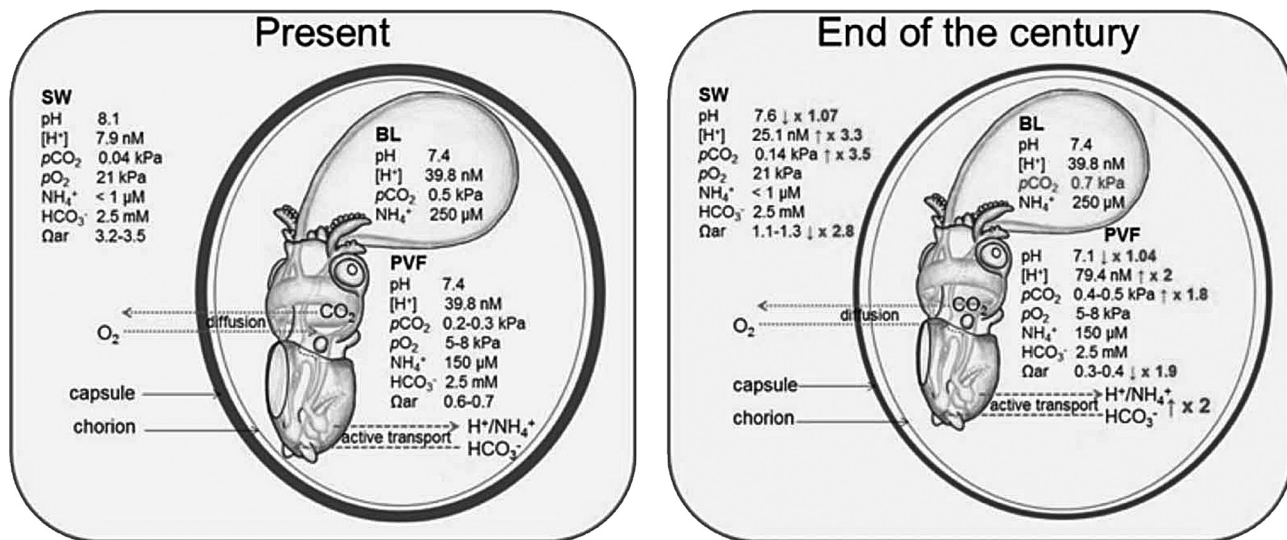


Fig. 1. – Abiotic changes in the microenvironment of *Sepia officinalis* embryos upon near-future seawater acidification scenarios. Acidification levels of pH 7.6 until the end of the century are predicted in the “worst-case” scenarios for ocean surface waters but can be already today experienced temporarily in coastal habitats. Due to the logarithmic pH scale a drop in seawater pH from 8.1 to 7.6 will already lead to a three-fold increase in hydrogen ions. The embryonic micro-environment is characterized by hypoxia, hypercapnia and high ammonia levels caused by a limited diffusion permeability of the protective egg capsule. Although increases in seawater pCO<sub>2</sub> are additive to the already high pCO<sub>2</sub> inside the egg capsule, the relative change in acidity [H<sup>+</sup>] and pCO<sub>2</sub> experienced by the embryo is smaller in the perivitelline fluid (PVF) compared to changes of surrounding seawater. It should be noted that the abiotic conditions of the PVF are influenced by environmental changes but also by the developing embryo itself. Depending on the physiological state of the embryo, metabolic end-products (e.g. NH<sub>4</sub><sup>+</sup>/NH<sub>3</sub>) and protons will be excreted at higher rates and will accumulate in the PVF. A modulation of the swelling process leading to a thinner egg capsule may further improve gas permeability in response to hypercapnic conditions. A smaller embryo in the near-future scenario indicates a developmental delay under elevated pCO<sub>2</sub> conditions. This figure also indicates growth. Seawater: SW; Blood: BL.

opment were less pronounced using the dry or wet mass but were more evident when using the mantle length of the developing embryo (Hu *et al.* 2011b). This suggests that elevated pCO<sub>2</sub> affects the size, in particular length, of the embryo, but to a lesser extent the total mass. In this respect, it needs to be mentioned that the aragonitic cuttlebone contributes approximately 4 % to the total body mass (Gutowska *et al.* 2010b, Dorey *et al.* 2013). As hypercalcification (for more details refer to section *Growth and calcification*) of the cuttlebone upon environmental hypercapnia significantly increased the weight of this internal calcified structure (Gutowska *et al.* 2010b), it is likely that reductions in body mass are partly masked by a heavier cuttlebone.

Although the phenomenon of reduced growth and development is very evident in cuttlefish and squid early life stages, the reasons for this phenomenon remain less well explored. It has been suggested that a hypercapnia induced developmental delay can be caused through different ways, including i) metabolic depression or ii) energy allocations or iii) a combination of both (Hu *et al.* 2011b, 2013, Stump *et al.* 2012, Pan *et al.* 2015).

On one hand, an uncompensated acidosis has been suggested to induce metabolic depression, and thus, hypercapnia-born reductions in growth and development in aquatic organisms (Langenbuch & Pörtner 2002, Pört-

ner *et al.* 2004). On the other hand, it was proposed that a higher fraction of energy is spent on acid-base regulation during environmental hypercapnia, leading to less energy available for growth and developmental processes (Stump *et al.* 2011, 2012, Hu *et al.* 2013).

A study by Rosa *et al.* (2013) could demonstrate significant reductions in metabolic rate at pH 7.5 and 22 °C in *S. officinalis* late stage embryos compared to animals kept under control conditions (pH 8.1). Furthermore, *S. officinalis* embryos exposed to hypercapnia levels of 0.4 kPa for 5 weeks responded with a general down regulation pattern of genes involved in energy consuming (e.g. acid-base genes) as well as energy providing (metabolic genes) processes in gill tissues (Hu *et al.* 2011b). Such a reduction of protein synthesis and down regulation of energy providing processes has been described as a general feature of organisms undergoing metabolic depression (Guppy & Withers 1999). These observations indicated that metabolic depression might play a role in developmental alterations during environmental hypercapnia in the cuttlefish *Sepia officinalis*. Nevertheless, more sensitive respiration measurements in combination with activity determinations of metabolic enzymes (e.g. citrate synthase) will be essential further research targets in order to provide a more definitive conclusion.



### *Epidermal ionocytes: embryonic sites for acid-base regulation*

During embryonic development of *Sepia officinalis*, rudimentary gill structures progressively develop and become complete when the organism has reached an adult-type morphology (Schippe *et al.* 1979, Hu *et al.* 2010). During cephalopod organogenesis, before systemic and branchial hearts are developed, contractile muscles of the yolk sac are responsible for convective circulation of hemolymph around the yolk syncytium and into the extensive lacunar blood system of the embryo (Portmann 1926). These blood sinuses are occupying large volumes of the embryonic body, particularly in the head region (von Boletzky 1987a, b). These morphological features prompted us to formulate the hypothesis that these epithelia are probably major sites for acid-base regulation, excretion and gas exchange in cephalopods prior to gill development.

Using immunohistochemical and *in situ* hybridization techniques, it has been demonstrated that in *S. officinalis* exactly these regions are characterized by  $\text{Na}^+/\text{K}^+$ -ATPase (NKA) rich, ion-regulatory cells that are scattered over the outer yolk epithelium (Hu *et al.* 2011a, Bonnaud *et al.* 2013). These ionocytes are further characterized by secondary active transporters involved in the secretion of protons such as  $\text{Na}^+/\text{H}^+$ -exchangers (NHEs) energized by the electrochemical gradient generated by the basolateral NKA (Hu *et al.* 2011a). Similar to the situation in *Sepia officinalis*, another cephalopod species, *Sepioteuthis lessoniana* has been demonstrated to exhibit epidermal ionocytes as well. These ionocytes were characterized in greater detail and have a basolateral orientation of NKA and V-type  $\text{H}^+$ -ATPase (VHA) and express  $\text{Na}^+/\text{H}^+$ -exchanger 3 (NHE3) in apical membranes (Hu *et al.* 2013). Using scanning selective microelectrode technique in combination with the NHE specific inhibitor ethylisopropylamiloride (EIPA) a dose dependent decrease of proton gradients on the yolk epithelium has been demonstrated. These findings indicate the importance of NHE-based proton secretion in these animals (Hu *et al.* 2011a). Moreover, acidified conditions (pH 7.3) triggered an increase in transcript levels of genes coding for transporters such as NHE3, VHA and a  $\text{Na}^+/\text{HCO}_3^-$  co-transporter (NBC) compared to animals kept under control conditions of pH 8.1 (Hu *et al.* 2013). These experiments indicated a role of these ion transporters and pumps in mediating acid-base homeostasis in cephalopod embryos during short-term acclimation to environmental acidification. However, it can be speculated that cephalopod embryos may not rely on a tight regulation of extracellular pH homeostasis to the same extent as adults do. Strong pH regulatory abilities of cephalopods were associated with oxygen transport efficiencies by the highly pH sensitive respiratory pigment, hemocyanin. However, embryonic stages were demonstrated to utilize a different composition of

hemocyanin isoforms, which are expressed differentially upon seawater acidification, indicating an adjustment of oxygen transport despite potential changes in blood pH (Strobel *et al.* 2012, Thonig *et al.* 2014). Unfortunately there is no study that measured blood pH in cuttlefish embryos exposed to different seawater pH to date. This information together with studies addressing the costs of acid-base regulation in early life stages would be highly relevant to investigate the concept of energy allocation during environmental hypercapnia in *S. officinalis*.

### *Juveniles and adults*

#### *Growth and calcification*

In contrast to early developmental stages of *S. officinalis*, juveniles and adults have been identified as relatively tolerant towards  $\text{CO}_2$  driven seawater acidification. For example, juvenile *S. officinalis* exposed to 0.6 kPa  $p\text{CO}_2$  (pH 7.1) maintained growth rates of approximately 4 % per day similar to control animals (Gutowska *et al.* 2008). This relative tolerance was accompanied by unchanged metabolic rates during short-term (24 h) exposure to the same acidification level (Gutowska *et al.* 2008). Also on the molecular level, no changes in gene expression of acid-base and metabolic genes has been observed in gill epithelia in response to short-term (2–11 days) as well as long-term (42 days) exposure to elevated seawater  $p\text{CO}_2$  levels of 0.4 kPa (pH 7.4) (Hu *et al.* 2011b). Although no changes in growth, metabolism and gene expression were observed, two studies demonstrated that calcification of the cuttlebone was impacted.  $^{45}\text{Ca}$  uptake increased and lamellar spacing decreased in response to environmental hypercapnia leading to a higher density of the chambered aragonitic cuttlebone in  $\text{CO}_2$  treated animals (Gutowska *et al.* 2010b, Dorey *et al.* 2013). The phenomenon of hypercalcification of internal  $\text{CaCO}_3$  structures in response to environmental hypercapnia has been also observed for fish otoliths and crustacean carapaces (Checkley *et al.* 2009, Ries *et al.* 2009) and was speculated to be the consequence of a chronic elevation of  $\text{HCO}_3^-$  levels in body fluids (Checkley *et al.* 2009, Gutowska *et al.* 2010b). Also in squid *Doryteuthis opalescens* the statoliths, which are essential for acceleration detection were found to be abnormally developed in high  $p\text{CO}_2$  (pH 7.55) treated animals (Navarro *et al.* 2014). It has been suggested that an altered density of the cuttlebone might negatively influence swimming behavior, active metabolism and prey capture. Preliminary studies could demonstrate that after an embryonic phase (63 days) hypercapnic conditions of 0.4 kPa  $\text{CO}_2$  evoked a reduced ability to catch prey and to launch attacks against prey organisms (Maneja *et al.* 2011). Similar behavioural defects in response to elevated seawater  $p\text{CO}_2$  levels were observed in the squid *Idiosepius pygmaeus* (Spady *et al.* 2014). Thus, future studies addressing the behavior of *Sepia officinalis* will represent

a fruitful research field in order to clarify the question regarding hyper-calcification-born behavioral defects under acidified conditions.

#### Acid-base physiology

*Sepia officinalis* has been described to have significant acid-base regulatory abilities to stabilize extra-cellular pH during environmental hypercapnia (Gutowska *et al.* 2010a). For example, in response to seawater hypercapnia of 0.6 kPa  $p\text{CO}_2$ , adult *S. officinalis* elevate their blood  $\text{HCO}_3^-$  levels from 3.4 mM to 10.4 mM within 48 h in order to buffer the excess of protons generated through the increased hydration of  $\text{CO}_2$  (Gutowska *et al.* 2010a). The cephalopod gill has been identified as the major organ that mediates this powerful compensation reaction, by actively pumping  $\text{HCO}_3^-$  into the blood and excreting protons or proton equivalents (Potts 1965, Hu *et al.* 2011b, 2014b). The cephalopod gill has evolved an acid-base regulatory machinery, that consists of ion transporters and pumps homologous to those found in vertebrate systems (Hu *et al.* 2010, 2011b, 2014b). A recent study demonstrated that similar to the situation in early life stages, the gill of adult cephalopods uses apical NHE proteins in order to secrete protons from the animal. The employment of NHEs can be regarded as an energetically favorable pathway for the secretion of protons due to high environmental and low intracellular  $\text{Na}^+$  concentrations, providing a natural driving force for the exchange of  $\text{Na}^+$  against  $\text{H}^+$  ions in marine species (Robertson 1949, Potts 1994). Furthermore, pioneer studies (Potts 1965, Schipp *et al.* 1979, Donaubaue 1981) already proposed that the gill of *S. officinalis* represents an important site for the excretion of nitrogenous waste products driven by the NKA. Recent studies using molecular and immunohistochemical techniques underlined these hypotheses and demonstrated the importance of  $\text{NH}_4^+$ -based proton secretion mechanisms in cephalopods. In this context, a special focus has been dedicated to the identification and involvement of Rhesus proteins in cephalopods, a group of channel proteins that were demonstrated to mediate  $\text{NH}_3$  transport in vertebrate and invertebrate systems (Gruswitz *et al.* 2010, Wu *et al.* 2010, Wagner *et al.* 2011, Henry *et al.* 2012, Hu *et al.* 2014b). Interestingly, a range of marine invertebrates including molluscs and echinoderms respond with increased  $\text{NH}_4^+$  secretion rates in response to simulated ocean acidification (Thomsen & Melzner 2010, Stumpp *et al.* 2012, Fehsenfeld & Weihrauch 2013, Hu *et al.* 2014a). As acid-base regulatory and excretory processes are directly linked (Hu *et al.* 2014b) it can be hypothesized that  $\text{NH}_4^+$ -based pH regulatory mechanisms represent an evolutionary ancient and conserved mechanism in many marine invertebrates.

#### Summary and future perspectives

The present review summarized the current find-

ings regarding the effects of ocean acidification on the cephalopod *Sepia officinalis*, and highlights the large difference in terms of sensitivity between ontogenetic stages. While adult cuttlefish seem to be relatively tolerant towards  $\text{CO}_2$  induced seawater acidification early life stages were demonstrated to be more vulnerable. Low pH, hypercapnia and high ammonia inside the egg capsule amplified by  $\text{CO}_2$  driven seawater acidification are probably the major abiotic stressors that negatively affect the development of the cuttlefish embryo. Here it should be noted that the abiotic conditions of the PVF are controlled by environmental factors as well as the embryo itself. Thus, future studies are needed to better understand the interplay of exogenous (by the environment) vs. endogenous (by the embryo) processes that affect the PVF chemistry. In particular the research on physiological responses of the embryo during exposure to hypercapnia, high ammonia concentrations and low pH represent important future tasks to fill important knowledge gaps. Although the recent advances in understanding the physiological responses of *S. officinalis* and other cephalopod species towards environmental hypercapnia shed light on some major questions, even more remains to be discovered.

I strongly believe that the application of new and powerful molecular techniques including the generation of transcriptomes, gene knock-down and *in situ* hybridization, etc., will quickly improve our understanding regarding regulatory mechanisms controlling the functional formation of regulatory epithelia. These epithelia enable *S. officinalis* to tolerate  $\text{CO}_2$  induced fluctuations in pH and allow for an embryonic development in an extreme microenvironment. This knowledge will help to better understand how environmental factors have shaped and will affect the physiology of *S. officinalis* in past and future oceans.

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